INTRODUCTION

Toxicology is the interdisciplinary branch of science deals with nature, effect and detection of poisons having following four principal sub divisions:

1. Environmental Toxicology
2. Economic Toxicology
3. Clinical Toxicology
4. Forensic Toxicology

Forensic Toxicology: Forensic toxicology is concerned with the medico-legal aspects of deleterious effects of chemicals on human and other animals.

Forensic Veterinary Toxicology is the sub division of Forensic Toxicology includes investigation of malicious poisoning to the animals, contamination of the food supply, insurance litigation and poisoning of wildlife. In recent years, law enforcement authorities became more cognizant about animal abuse and violence. Due to the increase of awareness in this concern more meaningful anticruelty laws have been legislated and there appears to be an increased willingness to investigate and prosecute cases of animal cruelty, including animal poisonings. .

Use of poison to kill wild animals for the various purposes has an old history. Human are killing wild animals for the purpose of meat (as food), medicine preparation, controlling population of animals, protecting crops, protect human-wildlife conflicts and to trading their body parts like ivory teeth (elephant), antler (deer), skin (tiger, leopard) and furs (bird) etc. as these have a high demand and cost in national international black market of various countries.

Earlier humans were using plant and animal poisons to kill wildlife animal but recently humans are using synthetic poisons such as carbamates, organochlorines, organophosphates, pyrethroids, anticoagulant and other types of rodenticides, compounds like phosphine, strychnine, paraquat, and cyanide are also used [2-6]. These poisons are easily available in market, silent in nature, more effective than traditional poisons, cheap and easy to administer than traditional poisons hence these types of poisons are becoming the preferred choice of the many wildlife animal killers. Intentional poisoning commonly

involves household poisons like rodenticides, insecticides and Nicotine etc. Agricultural pesticides and feed additives are involved in malicious livestock poisonings as these products are easily accessible in the farms. Natural toxins may be introduced to the expensive animals to be appearing accidental poisoning to attempt insurance claims. The selection of poison to kill animal varies region to region depending on accessibility of poisons.

The use of pesticide, insecticide, and other poisonous material has increased in the last few years for the purpose of protecting the crops in the agriculture field. The insects (pest) don't responds to older poisons after developing the resistance against it hence there is booming to introduce new synthetic poisons for the protection of crops [1]. At the same time these poisons are misused by animal killers leading to intentional, secondary and accidental poisoning of wild and domestic animals.

Methods adopted to kill the animal includes baiting carcasses with poisons, soaking grains in pesticide solution, mixing pesticides to form salt licks and poisoned waterholes tainting. Pesticides in liquid form may be injected into bait by use of a syringe [6]. Detection of these poisons in the poisoned baits and animal tissues is the recent challenge to many investigators working in the field.

The numbers of animal poisoning cases are receiving for identification of poison used in killing of animal. Many organizations are working in dealing with animal poisoning cases. The traditional methods of analysis are not suitable in such cases, hence chemical and instrumental technique has to be update for the detection of such poisons. [1]

Many analytical techniques are used to detect the poison residues in the animal tissues samples like vomit, gut contents material, faecal material, urine sample, blood sample, liver and kidney tissue, lung tissue and poisoned baits. Detection of these synthetic poisons from biological material as mentioned above of animals is biggest challenge for Forensic toxicologist. Many techniques have the limitations to detection these types of poisons. Instead, use of new and unusual poisons to kill the animal is another challenge at the recent time.

REVIEW OF LITERATURE

Many researchers carried out work for the analysis of toxicants used for animals poisoning. Some of them are following:

Brown et al. (1996) published details of analytical methods used for the determination of multiple pesticide residues and single pesticide residues in wildlife specimens submitted to the WIIS operating in England and Wales. The different methods involved a variety of sample clean-up and derivatisation techniques, combined with GC, GC/MS and/or High Performance Liquid Chromatography (HPLC) and covered a wide range of pesticides and their metabolites. The same author updated this review in 2005 and LC/MS and LC/MS/MS were included as frontline techniques for the determination of single pesticide residues (imidacloprid, paraquat and diquat), multiple (carbamate) pesticide residues and anticoagulant rodenticides [7].

Hunter et al. (2004) used LC/MS/MS (negative ion mode) for the quantitative determination of chloralose in specimens collected from suspected poisoned birds of prey. The method was compared to a gas chromatography (GC) procedure. LC-MSMS provided a simple and relatively quick analysis of the toxicant chloralose in animal tissues. Sample preparation can be limited to solvent extraction, dilution in HPLC mobile phase, and sample filtration. The selectivity of the determination eliminated the need for any sample clean-up, and the sensitivity achievable is more than satisfactory for the diagnosis of poisoning (or to confirm exposure) in animals [8].

Wang et al (2007) conducted retrospective study on the pesticide poisoning in domestic animals from 1999 to 2004 in the Austria. Author analyzed 380 suspected poisoning samples and among them 175 samples was confirmed for presence of poison. Among the pesticides found carbamate pesticides were most prominent followed by rodenticides anticoagulants and organophosphorous insecticides. These samples were characterized by HPLC technique using commercially available standards. Analysis was performed using tissue samples of lever of different species, gastric contents and poisoned baits. Presence of poison were performed by solid-phase extraction followed by chromatographic method (HPLC) using photo diode array detector. The study was conducted to determine the incidence and frequency of pesticide intoxication in animals in Austria to emphasize its relevance in veterinary practice for domestic animals [3].

Fernandez- Alba et al. (2008) reviewed advanced LC/MS based residue methods for the determination of multiple pesticides and their degradation products in foodstuffs which included animal tissues. Author described the advantages and pitfalls of these methods, particularly relating to large-scale screening, identification and quantitation of multiple pesticide residues. However, examples of the use of LC/MS/MS for routine monitoring of multiple pesticide residues that may remain in or on specimens taken from vertebrate wildlife or pets suspected of being accidentally or deliberately exposed to pesticides are rare [9].

Mol et al. (2008) utilised Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC/MS/MS) in the development of a generic extraction method for the simultaneous determination of pesticides, mycotoxins, plant toxins and veterinary drugs in feed and food matrices including meat [10].

Venkataramanan et al. (2008) analyzed case of malicious poisoning of Leopard in the Sandynallh reserve forest, India. In which Leopard was poisoned by use of carbofuran which was placed in the cow carcass. The poisoning was diagnosed by Thin Layer Chromatography in samples of stomach contents, intestinal loop and lever of Leopard carcass and muscles of cow carcass which was used to make preparation of bait. However poison was not detected in the kidney and lung specimens. A container cover was also recovered nearby the cow carcass which was helped to confirm the intentional poisoning in the case[11]

Cooper et al. (2009) discussed about dealing with the wildlife crime scene and proper use of tools and techniques and need of new technologies for overcoming the challenges in the field. As the wildlife crime has low social stigma causes delay in the response. Delay in the response to wildlife offence may result in the spoiling of evidences due to rain, wind, other climatic factors, human involvement and activity of other wild animals which results in greater challenge to investigate the evidences. Author suggested the use of equipments, latest tools and technology and scientific dealing with the wildlife crime scene, skill manpower to handle wildlife evidences and use of scientific protocol to minimize the risk of possible distortion of evidences. Author also suggested the interdisciplinary approach and exchange of data to solve the wildlife crimes [12].

Pang et al. (2009) combined gas chromatography mass spectrometry (GC/MS) and LC/MS/MS techniques following gel permeation chromatography (GPC) clean up, for the quantitative determination of several hundred pesticides in animal muscles (cow, sheep, chicken and rabbit). They concluded that LC/MS/MS was more sensitive than GC/MS for the determination of over 200 pesticides [13].

Kalaivanam et al. (2011) discussed about the case of secondary poisoning of a tiger in the Nilgiri Biosphere Reserve. Wild boars were the primary target of the regional people hence they laced the bananas with the phorate and poisoned the boars. Unfortunately carcass of the wild boar was eaten by the tiger and died. Sample of stomach, lever and kidney were sent to the RFSL for detection of any possible detection of poison. Laboratory conducted the TLC followed by GC-MS of the sample and confirmed the presence of phorate. The stomach contents also observed with the presence of blackish skin portion with the hairs which was latter confirmed is of wild boar. Hence it was confirmed that tiger died due to consumption of wild boar carcass which was poisoned by use of phorate [14].

Novotny et al., (2011) published details of case studies involving carbamate intoxication of animals in the Czech Republic The authors only noted the use of 'chromatographic techniques' for the analysis of tissue (liver), solids (bait, unidentified material) or liquid samples (gastric content). Samples were extracted by petroleum ether and after evaporation on the rotary vacuum evaporator and dissolution in isooctane were analyzed by the gas chromatography method with a nitrogen-phosphorous detector. The analysis was conducted at the State Veterinary Institute in Jihlava Czech Republic [15].

Bell L.S. (2011) reviewed and discussed the techniques used in the wildlife which includes morphological methods (Osteology, microscopy, necrospy) and chemical methods specifically used in poison detection. In chemical methods author emphasized that validation and verification of poisons are possible due to extensive chemical reference libraries but the physiology of different species and the degradation metabolites can make identification more difficult. To overcome this challenge author suggested updating the available technologies and methods. Authors also discussed about the use of pesticides in different wildlife cases including intentional and secondary poisoning [16].

Gallocchio et al. (2014) examined the 561 suspected baits samples of baits, lever and stomach contents at the Istituto Zooprofilattico Sperimentale delle Venezie (North-Eastern Italy) in the period of 2012-2013. Samples were analyzed by a method based on a combination of liquid chromatography with diode array/fluorescence detection (HPLC-DAD/F). The proposed HPLC method with DAD/F to qualitatively identify and determine eleven anticoagulant rodenticides has proven to be effective and fit-for-purpose in the investigation of animal poisoning incidents where Anticoagulant Rodenticides are suspected to be the toxic agent. Method successfully identified 11 different AR (brodifacoum, bromadiolone, chloropahacinone, coumachlor, coumafuryl, coumatetralyl, difenacoum, diphacinone, flocoumafen, pindone, warfarin). Author also studied qualitative parameters such as limit of detection, specificity, carryover and sample stability for three matrices (liver, stomach content, bait), showing that the method was suitable for the simultaneous detection of the eleven AR in these matrices. The assay is fast, requires small samples and simple extraction procedures, hence author suggested application of method in routine analyses for characterization of anticoagulant rodenticides in suspected cases of animal poisoning [17].

Ogada (2014) reviewed the pesticide poisoning in wildlife of Africa. In this review he discussed about the various poisons which is used for killing the wild animal which includes plant based poisons like opium, tephrosia spp, mundulia) and synthetic poisons like organophosphate, organochlorine, Carbamate (carbofuran), pyrethroid etc. The author also discussed about the methods used to kill the animal like poisoning the baits (fruits, breads, meat etc.), use of waterholes and poisoned arrows. Author emphasized on the reasons which are driving the wildlife animal killing like huge demand of animal body parts in the illegal market for preparation of traditional medicines , making of ornaments and for food purposes. Author emphasized on the use of new synthetic poisons to kill the animal as these are easily available, cost efficient and effective than the traditional poisons which were used earlier by the wildlife killer. Author suggested some preventive measures to control the wildlife killing which includes the banning of poisons regulation and control of distribution, development of forensic protocol for detection of such cases, development of regional pesticide centres, strengthening the enforcements and enhancement of penalties in the same aspects [6].

Bille et al. (2016) discussed about the records referring to animal poisoning and poisoned baits cases between the period of 2007-2013. Total 698 suspected poisoned baits were received and among them 292 baits (41.8%) sample were confirmed for presence of poison.

Author also noticed the seasonal trend in poisoning. Anticoagulant redenticides found the most prominent cause for animal poisoning by baits. Author emphasized on the widespread malicious poisoning in the north eastern Italy by using agricultural pesticides but at the same time use of non agricultural poisons became the challenge for detection. Author suggested the up gradation in similar context [18].

Sell et al., (2017) reported new analytical multiclass method based on QuEChERS technique, which has proven to be effective in diagnosing fatal poisoning cases in animals. Method was developed for the determination of analytes in liver samples comprising rodenticides, carbamate and organophosphorus pesticides, coccidiostats and mycotoxins. The procedure includes addition of acetonitrile and sodium acetate to 2 g of homogenized liver sample. The mixture was shaken intensively and centrifuged for phase separation, which was followed by an organic phase transfer into a tube containing sorbents (PSA and C18) and magnesium sulfate, then it was centrifuged, the supernatant was filtered and analyzed by liquid chromatography tandem mass spectrometry. A validation of the procedure was performed. Repeatability variation coefficients <15% have been achieved for most of the analyzed substances. Analytical conditions allowed for a successful separation of variety of poisons with the typical screening detection limit at ≤10 μg/kg levels. The method was used to investigate more than 100 animals poisoning incidents and proved that is useful to be used in animal forensic toxicology cases [19].

Radhakrishnan (2018) studied the wildlife mortality in Periyar Tiger Reserve, Kerala, India of the period January 2011 to March 2013. Author discussed about the some cases where differential diagnosis were identified. Author discussed poisoning cases which includes carbofuran in bonnet macaque, warfarin in wild boar, endosulfan toxicity in gaur and imidacloprid toxicity in Asian elephant within the region. Author emphasized that there is no literature that identifies the specific poisons or chemicals involved in wildlife animal poisoning cases from the state of Kerala on the five data bases namely PubMed, Web of Science, Scopus, Ovid and ProQuest including google Scholar using the search term wildlife, poison and Kerala. Identification of poison is the first step to helping the government agencies to implement the regulatory measures. Author reported use of specific poisons detected in wildlife mortality events in Kerala of two years [20].

RATIONALE FOR THE STUDY

Cases related to animal poisoning are increasing nowadays. Only some of the laboratories are handling these cases, however due to lack of expertise and specialized analytical facilities, laboratories are getting delay for the report. For this reason there is need to develop a method for identification of toxicants in the cases of animal poisoning.

OBJECTIVES OF THE STUDY

1. To analyze toxicants from various samples (Viscera, Blood etc.) using Planar Liquid Chromatography i.e. TLC/ HPTLC etc.
2. To develop direct injection method for the analysis of toxicants from animal biological fluids i.e. blood etc.
3. Application of developed method in spike and real samples.

METHODOLOGY

Ethical Clearance: Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) will be followed for the research work (Schedule Y. [Last accessed on 2010 Jan 02]).

Selection of Toxicants: Commonly used pesticide for the poisoning of animal i.e. Carbamate (Carbaryl, Carbafurone), crmidine, malathion and plant alkaloid strychnine etc. will be selected for the study.

Collection of Samples: For spike samples analysis goat meat will be purchased from butcheries and blood of animals (herbivorous as well as carnivorous) will be bought from Veterinary Blood Bank.

Real Samples: On request real samples will be obtained from Wildlife Institute of India (WII), Dehradun, Indian Veterinary Research Institute (IVRI), Bareilly etc.

Selected Analytical Method: Liquid Chromatographic methods will be used for analysis

Planer Chromatography: TLC/HPTLC will be used for the analysis of toxicants from viscera.

Column Chromatography: MLC is an alternative technique to conventional reversed phase liquid chromatography in which the mobile phase is an aqueous solution of a surfactant at a concentration above its critical micellar concentration, that is, in a medium where micelles exist. In micellar liquid chromatography, the optimization of the separation conditions is usually carried out by using mobile phases at fixed pH, which is selected in turn performing several previous runs at fixed concentration of surfactants and organic modifier and varying pH.

The accurate and reproducible elution behavior of compounds with micellar mobile phases has permitted the development of two-factor (surfactant and modifier) optimization strategies that expedite the experimental work. The main advantages of MLC are the lower cost and toxicity, the biodegradability of the solvents used, and the easy dissolution of analytical samples.

One of the major advantages of MLC is the capability to describing the retention behaviour of compounds eluted with hybrid micellar mobile phases of surfactant and organic modifiers to a high degree of accuracy.

Method Development: Method will be developed using standard of toxicants.

There are following steps involved in the development of direct injection method (MLC) for the analysis of toxicants.

1. To optimize experimental condition for the separation of different toxicants. This will include the range of surfactant and modifier to be used, and also the selection of pH.
2. To obtain the chromatographic parameters for the substances to be separates. This will be achieved by using integrating peak parameters to obtain the retention time, efficiencies and the asymmetry.
3. On the basis of maximum resolution and minimum analysis time optimum mobile phase will be selected.
4. To obtain the best analytical characteristics like calibration, limit of detection (LOD), repeatability, reproducibility, selectivity etc.

5. Comparison of the obtained result with already developed TLC/HPTLC/HPLC methods.

Applications of the developed method: Analysis of toxicants in spike and real samples of blood and other physiological fluids will be carried out.

EXPECTED OUTCOME OF THE PROPOSED WORK

Sample preparation steps in many applications constitute the main part of the total bio analytic method in various laboratories with a large number of routine samples for chemical analysis. It means that a lot of time must be devoted to sample work-up procedures. This is largely the reason why much effort has been put into the development of HPLC systems that can tolerate the direct injection of physiological fluids. The standard protocol for the analysis of poisons will be developed in this study which could be adopted by various organisations i.e. Forensic Science Laboratory, Wildlife Institute of India and Veterinary Institute etc. for monitoring the animal poisoning cases.

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TENTATIVE PROPOSED CHAPTER OUTLINE

1. Introduction
2. Review of Literature
3. Methodology
4. Results and Discussion
5. Conclusion
6. References